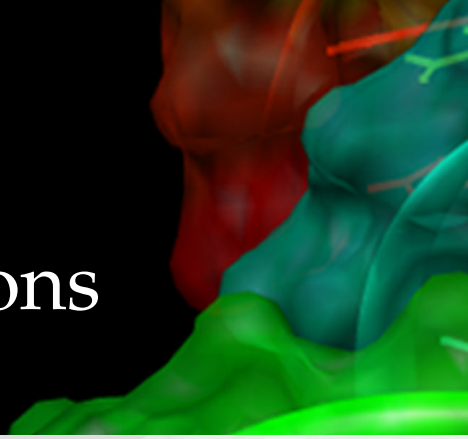
RNA Rules:
The Many Faces and Functions of Ribonucleic Acids



Long overshadowed by its more famous DNA cousin, RNA is enjoying a molecular renaissance. RNA serves as a middleman between DNA templates and protein machinery. However, RNA molecules have catalytic activity of their own (a discovery for which Thomas Cech, Ph.D., and Sidney Altman, Ph.D., won the Nobel Prize in Chemistry in 1989). And small segments of RNA can silence the gene expression of their brethren (a discovery for which Craig Mello and Andrew Fire won the Nobel Prize in Physiology or Medicine in 2006). Researchers are even learning how to create entirely new types of RNA molecules. Today, CCR investigators are exploring and reinventing the roles of RNA in health and disease.

"For years, people thought of RNA as a not very interesting molecule," said Bruce Shapiro, Ph.D., Senior Investigator in CCR's Basic Research Laboratory. "Essentially, there was the central dogma of going from DNA to RNA to protein; and, RNA was just an intermediary. Then a revolution started. Much of what people used to call junk DNA is in fact transcribed, conferring whole new levels of cellular control."

A new RNA Biology Initiative is bringing together CCR investigators to exchange ideas and expertise on the structure, function, and biological roles of RNA. Through workshops, shared technology development, collaborations, and mentorship, the Initiative is building on the strengths of CCR's ongoing research in this influential and rapidly evolving area of biomedical research.

Errors in Transcription

Even the processes supporting basic RNA transcription from DNA are more complicated than foretold. Mikhail Kashlev, Ph.D., Senior Investigator in CCR's Gene Regulation and Chromosome Biology Laboratory, has uncovered novel disease-promoting mechanisms by focusing his research on the molecular interactions that support efficient RNA synthesis. "We really want to know the role of transcriptional fidelity in cell physiology," said Kashlev. "This is a very under-investigated question."

When RNA polymerase II (Pol II) is recruited to the promoter of a gene to be transcribed, it does not rush to get the job done. Instead, it pauses first, in so-called promoter proximal elongation arrest. This pause in transcription may last for just a second or it may last for hours. "This is very important because the enzyme is thereby poised for a quick transcriptional response to additional signals," said Kashlev. "It is also important to coordinate the timing of transcriptional elongation for alternative splicing."

Kashlev's laboratory uses biochemical approaches to discover elements in DNA (*cis* acting signals) that modulate transcriptional arrest and, in collaboration with others, has analyzed factors that bind DNA (*trans* acting signals) to similarly modulate the process. They have developed techniques to reconstitute the elongation process *in vitro*, with the purified polymerase, synthetic DNA, and RNA oligonucleotides. "We can reconstitute the elongation of any sequence you want with up to 90 percent efficiency," said Kashlev.

Recently, his laboratory discovered that in addition to *cis* and *trans* factors, transcriptional

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(Photo: R. Baer)

Maria Kireeva, Ph.D., and Mikhail Kashlev, Ph.D.

errors can strongly arrest Pol II. The erroneous incorporation of a single ribonucleotide creates a mismatch between the DNA template and the growing RNA molecule, resulting in a physical disturbance to the active site of Pol II. "Elongation can't continue until the error is removed," said Kashlev. "But Pol II has a very limited ability to excise errors." Left uncorrected, paused elongation complexes could litter the genome.

This finding gave Kashlev and his Postdoctoral Fellow, Masahiko Imashimizu, Ph.D., a way to study transcriptional fidelity *in vivo*, where the transcriptional error rate is normally very low—on the order of 1 in 10,000 to 1 in 100,000—making it difficult to isolate these instances. "What if instead of sequencing all the mRNA isolated from a cell, we isolated all the elongation complexes that are paused?" asked Kashlev. "We should be able to enrich our population of mRNAs with errors." Isolating all the complexes from *E. coli* and performing high-resolution sequencing of the RNA to look for errors at the 3′ nascent end of the mRNA, Kashlev discovered that about one percent of all pausing events were due to errors.

"When errors arrest transcription very strongly, the genes are not expressed," said Kashlev. "Even more importantly, errors can also generate problems for DNA replication. When DNA polymerase collides with the transcriptional fork, replication is aborted. If that happens again, a double-stranded DNA break occurs."

Somewhat paradoxically, error-prone transcription can be beneficial for the cell. Celine Walmacq, Ph.D., a Postdoctoral Fellow, and Maria Kireeva, a Staff Scientist who initiated investigation of transcriptional fidelity mechanisms in Kashlev's laboratory, established that mutations in the Pol II catalytic subunit, rendering transcription fast and error-prone, reduce transcription arrest at UV-induced DNA lesions *in vitro* and increase cell resistance to UV irradiation.

The discovery of several classes of mutations in Pol II that alter transcriptional fidelity and mismatch extension by Kashlev's lab in collaboration with Zachary Burton, Ph.D., Professor, Michigan State University, and Jeffrey Strathern, Ph.D., Chief of CCR's Gene Regulation and Chromosome Biology Laboratory, opens new research directions. Indeed, errors in RNA transcription have been proposed as a route to cell pathophysiology. But the assumed mechanism has been transcriptional errors propagating to a particular protein, for example, transcriptional errors leading to mutated DNA polymerase, which in turn would produce faulty DNA replication.

"People have suggested that low fidelity transcription may be a priming event in cancer," said Kashlev. Such a mechanism is very hard to prove because it would occur at the individual cell level before any cancerous transformation. Kashlev's work suggests that errors in nascent mRNA may have an important direct effect on DNA through transcriptional arrest, an effect that may become even more important in the deteriorating cellular conditions of cancer or aging.

Viral RNA Dynamics

Viruses co-opt their hosts' transcriptional machinery to replicate. Retroviral RNA is reverse transcribed into DNA, which integrates into the host genome. The DNA is then transcribed to create more viral RNA, which is exported from the nucleus to join its protein cohort in the formation of a new virus. During DNA synthesis, genetic information from both packaged RNAs can be combined and genetic recombination becomes clinically significant as the viral progeny can acquire new properties and vulnerabilities. Wei-Shau Hu, Ph.D., Senior Investigator in CCR's HIV Drug Resistance Program Retroviral Replication Laboratory (RRL), wants to know exactly what happens to HIV RNA within the environment of a cell.

While studying recombination, Hu realized that a number of basic facts about viral RNA packaging were unknown or based on untested assumptions. For example, how many viral RNA molecules are

(Photo: R. Baer)

Olga Nikolaitchik, Ph.D., Wei-Shau Hu, Ph.D., and Jianbo Chen, Ph.D.

packaged in an individual HIV virus? If you take 100 virus particles, how many contain viral RNA at all?

The prevailing answers were based on biochemical data. "When you do a biochemical study, you know the amount of protein and the amount of RNA," explained Hu. "You correlate these numbers and assume an even distribution. But there are error rates in both measurements, which lead to big differences in predicting how many of those particles have RNA genomes and can be infectious."

Staff Scientist Jianbo Chen, Ph.D., working with Research Biologist Olga Nikolaitchik, Ph.D., took the lead in designing a reporter system based on RNA-binding proteins to detect viral RNA in a single viral particle. This involved directly engineering a stem-loop structure into the HIV genome that could be bound by a fluorescent reporter.

"We discovered that HIV is really quite good at packaging two, and only two, copies of the RNA genome per virus," said Hu. "It's a highly regulated process. But, how?"

Nikolaitchik led a study to first distinguish between two hypotheses: regulation based on RNA mass versus number of molecules. She generated viral genomes of dramatically different sizes and showed that regardless of size, the virus always packaged two copies of the RNA genome. The RNA molecules of the viral genome do not anneal to one another through base-pair complementarity along the entire molecule the way DNA does; instead, they have been shown to pair off through a short dimerization sequence affixed to one end of each molecule. When Nikolaitchik engineered RNA that could self-dimerize, she found that only a single copy was packaged.

"We are very interested in finding out exactly when and where these RNAs find each other and dimerize," said Hu. Her laboratory has shown that this must happen in the cytoplasm, not the nucleus. Furthermore, full-length viral RNA can exploit host nuclear export pathways to exit the nucleus, but Hu and her colleagues have shown that viral RNAs that use different export pathways do not recombine nearly as well.

To continue studying RNA sorting and packaging, Chen is developing a system to visualize its transport through the cytoplasm in living cells to its assembly site at the plasma membrane. "The live-cell imaging is technically very challenging," said Hu. "RNA moves extremely fast; you need fast cameras (25 pictures/sec) to capture its movement. But, thanks to Jianbo's efforts now we can track a single molecule of viral RNA moving inside a cell. Because HIV uses the host machinery to transport RNA, these studies also help us understand how human cells transport cellular RNAs."

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Viral Havoc

Joseph Ziegelbauer, Ph.D., Tenure-Track Investigator in CCR's HIV and AIDS Malignancy Branch, focuses on the role of RNA in the lifecycle of a DNA virus, the Kaposi's sarcoma-associated Herpesvirus (KSHV). After infecting cells, these herpes viruses can exist quietly in a latent phase, remaining under the immunological radar by expressing only a handful of protein encoding genes while expressing an abundance of miRNAs. Like endogenous miRNAs, these viral miRNAs modulate cellular gene expression, presumably in the service of increased viral survival and propagation.

"We are trying to use these miRNAs to tell us humans what the virus already knows about genes that are important for infection," said Ziegelbauer.

Identifying the cellular targets of viral miRNAs is more complicated than it might seem. Bioinformatic approaches to look for sequence complementarity between miRNAs and potential targets turn up many false positives, relying as they do on very small segments of nucleotide

pairing. False negatives also arise when the miRNA operates despite imperfect complementarity.

As a postdoctoral fellow in the laboratory of Don Ganem, M.D., at the University of California, San Francisco, Ziegelbauer began studying the recently discovered viral miRNAs by examining gene expression patterns in cells infected with the virus. When he joined CCR in 2008, he brought these data with him and has since expanded his search for significant viral miRNA targets.

"Our approach has been to use multiple ways of either introducing or inhibiting the miRNA and then integrating those data sets to find the genes that are changing the most across different experiments," said Ziegelbauer. "When we started, we were looking at a short list of 50 predicted targets."

Many of these targets are responsive to more than one of the 18 individual miRNAs encoded by the herpes virus. "Redundancy is a central theme, and that gives us another way to filter these targets. We take redundancy to mean that the target is potentially important and worthy of follow-up."

More recently, Ziegelbauer has been using network analysis to examine how the target genes are interacting with one another as an additional tool for identifying the important players and forming hypotheses about their cellular roles.

"I'm really excited about the next steps, one of which is a collaboration with the trans-NIH RNAi facility," said Ziegelbauer. "They are testing knock downs of some of our targets to figure out how they affect particular phenotypes of infection. They do the hard work of optimizing your assay for an RNAi screen and then they conduct the screen. That would be really difficult for us to do on our own."

Ziegelbauer is also collaborating with his Branch colleagues to use proteomic approaches on fresh clinical samples. "In our Branch, there are clinicians treating patients with KSHV infections," said Ziegelbauer. "It gives us a way to learn about the biology of the disease directly."

Using two-color quantitative immunoblotting, Ziegelbauer can look at vastly different levels of protein abundance in individual samples. Proteins give yet another view into the function of miRNAs and they may reflect a more faithful view of their function. Ziegelbauer recently published a study in *PLOS Pathogens* highlighting viral miRNA targets implicated in angiogenesis and immune evasion.

"I wouldn't ever rely on just the clinical or just the cell culture data," said Ziegelbauer. "But if we can show a miRNA target is repressed in a cell culture and we then look at uninfected versus infected lymph nodes and see the same target gene is repressed, they are pieces of the puzzle that can be put together to say that these genes are really repressed in patients."

RNA on the Offensive

Shapiro has learned a lot about the fundamentals of RNA structure and function, since he began his RNA research in the 1980s. Now, he is using that knowledge to build nanostructures out of RNA that Mother Nature has not yet dreamed of. He wants to then put those structures to use in curing disease.

"RNA is interesting because it can naturally fold into fairly complex

(Photo: R. Baer)

Left to right: Anna Serquiña, M.D., Ph.D., Joseph Ziegelbauer, Ph.D., Xiaoyan Liu, Ph.D., Christine Happel, Ph.D., and Dhivya Ramalingam, Ph.D.

(Photo: R. Baer)

Bruce Shapiro, Ph.D., and Kirill Afonin, Ph.D.

structures. It can serve as a catalyst or as an information storage medium. It has lots of natural functionality. And you can control the self-assembly process by understanding base-pairing interactions and known structural geometries," said Shapiro.

To define the elements of RNA structures, Shapiro and his colleagues, Eckart Bindewald, Ph.D., and Wojciech Kasprzak, M.S., went to the Protein Data Bank (PDB), a database of molecular structures that is more expansive than its name implies, and extracted three-dimensional "motifs" that are found among the known RNA structures. "We ultimately identified about 13,000 motifs and we view them like LEGO blocks that we can piece together to generate the types of 3-D patterns we want," said Shapiro. These motifs are freely available online at the RNA junction database that they produced (http://rnajunction.abcc.ncifcrf.gov). Shapiro's group also developed software to aid in the nanoscale structure design and in the design of sequences that can self-assemble into nanostructures.

With his Research Fellow Kirill Afonin, Ph.D., Shapiro has experimentally created some of these computationally designed shapes. One is a nanometer-scale hexagon formed from individual elements shaped like dumbbells with so-called "kissing loops" of RNA on each end. In collaboration with Luc Jaeger, Ph.D., from the University of California, Santa Barbara, they experimentally verified the formation of these hexagonal structures and by programming each kissing loop differently, they gained greater control over the assembly and purity of these hexagonal shapes. They have also experimentally formed cubes from either 6 or 10 computationally designed motifs, so that the individual strands do not self-fold. They can verify the shapes through atomic force microscopy, or cryo-electron microscopy but they have also incorporated specialized RNA molecules—aptamers—that they engineered to fluoresce when pieces come together properly.

As much fun as building nanostructures from RNA can be, this research is not merely academic. Shapiro is driving this work towards novel therapeutic applications.

RNAi is both a naturally occurring phenomenon and a research tool for silencing the translation of specific genes. It may also be a very powerful tool for therapeutically altering gene expression in disease. One difficulty has been delivery of the siRNAi agents, which are easily degraded by nucleases. Shapiro has shown that he can attach multiple siRNA molecules to the RNA nanostructures his group has created. These nanodelivery vehicles enter the cell intact, whereupon the siRNAs are cleaved by an enzyme (Dicer) to become functionally active. Experimentally, he has shown that siRNA delivered via the nanostructures can silence the expression of the reporter gene—*EGFP*—in cell culture and in xenograft mouse models.

In addition to a variety of pure RNA structures, Afonin and Shapiro have also experimented with DNA/RNA hybrid structures that confer added stability and functionality. They have shown, in collaboration with Eric Freed, Ph.D., Senior Investigator, RRL, that the activated forms have the ability to silence targeted HIV. Also, they have silenced multiple cancer targets in cells and in xenograft mouse models. "We have a database of structures that are computationally designed, but have not yet been tested experimentally," said Shapiro. A lot of the work the group has done so far validated the fundamental concepts of design, self-assembly, and delivery of functional RNA nanoconstructs, but Shapiro says, "We are working towards targeting specific cancer pathways and viral diseases with these designer molecules."

To learn more about Dr. Hu's research, please visit her CCR website at http://ccr.cancer.gov/staff/staff.asp?name=hu.

To learn more about Dr. Kashlev's research, please visit his CCR website at http://ccr.cancer.gov/staff/staff.asp?name=kashlev.

To learn more about Dr. Shapiro's research, please visit his CCR website at http://ccr.cancer.gov/staff/staff.asp?name=shapiro.

To learn more about Dr. Ziegelbauer's research, please visit his CCR website at http://ccr.cancer.gov/staff/staff.asp?name=jziegelbauer.